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14. ABSTRACT

In this report we give a preliminary account on the consequences of mammary-specific Focal Adhesion Kinase (FAK) deletion in mammary-specific polyoma middle-T transgenic mice. We monitored mammary carcinogenesis in positive control (FAKFlox/Flox; MMTV-PyVT) and target (FAKFlox/Flox; MMTV-Cre; MMTV-PyVT) females. We found that mammary-specific FAK deletion lengthens the tumor-free interval by 33.2 days. We also found that cumulative subcutaneous mammary tumor burden and tumor growth rate were larger in positive control females than in target females. Additionally we also found that FAK is virtually not expressed in mammary tumors from target animals, but FAK expression was abundant in positive control animals. Transgenic polyoma middle-T antigen expression was similar in mammary tumors from both positive control and target females. Based upon the preliminary data, we conclude that FAK, although not absolutely required, but it still plays a contributory role in polyoma middle-T antigen induced mammary carcinogenesis in female mice.

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Introduction

Human breast cancer is a multistep neoplastic process [1], during which integrins are indispensable mediators from the initial neoplastic transformation [2], through local invasion [3] and in the metastatic spread [4]. The focal adhesion kinase (FAK), one of the major mediators of integrin signaling [5], has been implicated both *in vitro* and *in vivo* in the development of human breast cancer [2], [6], [7], [8].

Originally I proposed to create a doxycycline-inducible transgenic expression of a novel, FAK-inhibitory protein called FIP200 [9], [10]. Due to technical difficulties with transgene expression, I had to modify my research approach. In my new approach I decided to directly delete FAK in the mammary epithelium of mice employing the Cre-loxP method [11]. The genetically modified mouse strains were already available in our lab. In my midterm report I gave a detailed analysis of virgin and lactating female mice in which FAK was specifically deleted in the mammary epithelium. No morphological abnormalities were found in the mammary gland of virgin mice however, lactating mice have severe lobulo-alveolar hypoplasia in the mammary gland.

After completing the analysis of postnatal (pregnancy-associated) development of the mammary gland, we focused our attention on the relationship between mammary carcinoma and FAK. In a previous report [2] it was found that normal, non-neoplastic human mammary epithelium has minimal FAK immunoreactivity in immunohistochemical studies, whereas invasive lobular carcinoma or ductal carcinoma *in situ* has strong FAK immunoreactivity. Moreover, adjacent to areas of invasive mammary cancer, histologically normal mammary epithelium has increased FAK immunoreactivity. The authors in the report therefore argued that FAK overexpression is an early event in mammary carcinogenesis and is not restricted to fully invasive neoplasia. Studies from our lab showed that FAK is indispensable for robust cell proliferation [12]. Moreover, FAK inhibits endocytosis of MT1-MMP (a membrane-bound matrix metalloproteinase) and thereby promotes degradation of the extracellular matrix [13]. Based upon these observations, we hypothesized that FAK signaling is important in mammary carcinogenesis and possibly metastasis. To test our hypothesis, we introduced mammary epithelial-specific deletion of FAK into MMTV-PyVT transgenic mice which have an early-onset of metastatic mammary carcinoma due to mammary epithelial-specific expression of the polyoma virus middle-T antigen (PyVT). We expected that the incidence of primary mammary carcinoma, as well as the incidence of metastatic disease, will be significantly lower in FAK conditional knock-out MMTV-PyVT females than in FAK wild-type MMTV-PyVT females.

Body

Aim 3. Effects of FAK deletion on mammary carcinogenesis, Months 25-36

a. *Transgenic carcinogenesis – preliminary studies.*

a. *Procurement of MMTV-PyVT transgenic animals.*

b. *Analysis of FAK expression and FAK phosphorylation in the primary mammary tumors and in the metastatic foci (if any) in the MMTV-PyVT transgenic animals.*

Rationale

To experimentally induce mammary cancer in mice, one has two major options. One is chemical carcinogenesis after *per os* treatment with dimethylbenz[*a*]anthracene (DMBA) [14], the other one is transgenic carcinogenesis. Among the transgenic mouse models [15], [16], one has several choices. The *pros* and *cons* of each system should be weighed and one system has to be chosen according to experimental requirements. Among the various transgenic systems we chose the MMTV-PyVT (also called as MMTV-PyMT) [16] transgenic line, because it develops metastatic mammary cancer early in life. In this transgenic line the expression of polyoma virus middle-T antigen is directed to the mammary epithelium due to the activities of the MMTV (Mouse Mammary Tumor Virus) promoter.

It is also important to analyze MMTV-PyVT-induced mammary carcinoma for FAK expression and activation to see if FAK is overexpressed in these tumors similar to human breast cancer.

Materials and Methods

MMTV-PyVT transgenic mice were obtained from the Jackson Laboratory (Bar Harbor, ME). This transgenic line was maintained by crossing MMTV-PyVT transgenic males to wild-type FVB females. (The early onset mammary carcinogenesis in transgenic females renders these affected females unable to lactate).

Our lab has produced a genetically modified mouse strain in which the third coding exon of both *FAK* alleles is flanked by two unidirectional *loxP* sites ($FAK^{Flox/Flox}$ mice, floxed mice). Cre-mediated deletion of exon 3 leads to a frame-shift mutation due to direct splicing from exon 2 (which contains the ATG initiation codon) to exon 4, and the resulting protein will be truncated (~70 aa) and nonfunctional (lacking the majority of FAK sequences) [17]. The MMTV-*Cre* mouse strain was obtained from the National Cancer Institute (Frederick, MD).

All mice were housed in the Transgenic Mouse Core Facility at the College of Veterinary Medicine, Cornell University (Ithaca, NY) under specific pathogen-free conditions in accordance with institutional guidelines. All experimental procedures were approved by Cornell University's Institutional Animal Care and Use Committee.

DNA isolation from the tail of the offspring have been described elsewhere [18]. FAK alleles were genotyped in a Polymerase Chain reaction (PCR) using the following primer pair: P1, 5'-GCTGATGTCCCAAGCTATTCC-3' and P3, 5'-AGGGCTGGTCTGCGCTGACAGG-3'. Using this primer combination the PCR reaction amplifies 1.6-kb, 1.5-kb, and 550-bp fragments from the $FAK^{flox/flox}$, wild-type, and FAK deleted alleles, respectively after 30 cycles of 94°C (3 min), 67°C (2 min), and 72°C (4 min). Primers CreF, 5'-CGCAGAACCTGAAGATGTTCGCGATTA-3' and CreR 5'-TCTCCCACCGTCAGTACGTGAGATATC-3' were used in another PCR reaction, which generated a 400-bp fragment after 35 cycles of 95°C (30 s), 60°C (30 s), and 72°C (30 s) in samples were the Cre transgene was present [17]. Transgenic and wild-type polyoma middle-T alleles were detected using the following primers and conditions. Primers P1 (5`- CAA ATG TTG CTT GTC TGG TG -3`) and P2 (5`- GTC AGT CGA GTG CAC AGT TT -3`) amplify the wild-type allele (200-bp), whereas primers P3 (5`- GGA AGC AAG TAC TTC ACA AGG G -3`) and P4 (5`- GGA AAG TCA CTA GGA GCA GGG -3`) recognize the transgenic allele (400-bp). The following conditions were used with P1-P4 primers: 25 cycles of 58 ºC (30 s), 72 ºC (35 s), and 72 ºC (120 s). The PCR products were resolved in 1.2% agarose gels containing ethidium-bromide and the specific bands were identified by size against molecular weight markers.

MMTV-PyVT transgenic mice were observed and palpated weekly for the presence of mammary tumors starting at 8 weeks of age. Tumor-bearing female mice were euthanized once tumors were interfering with normal locomotion, the animal showed signs of respiratory distress, or weight loss was noted. Euthanasia was performed using CO_2. After euthanasia the animals underwent complete necropsy and tissues, along with the rest of the carcass, were fixed in 4% formaldehyde at 4 ºC for 16 hours. After fixation, the tissues were first washed in PBS (3 times, 20 minutes each), transferred to 65% ethyl-alcohol (30 minutes) and finally moved to 70% ethyl alcohol for long-term storage at room temperature. The formalin-fixed tissues were embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined histologically using an Olympus BX41 microscope. Photomicrographs were taken with an Olympus DP70 camera.

During necropsy, in addition to collecting samples for histology, mammary tumor and lung samples were collected to prepare protein extracts. Organs were first snap-frozen in liquid nitrogen and then were ground using a mortar and a pestle. Finely ground tissue was lysed at 4 ºC for 15 minutes in RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100) supplemented with protease inhibitors (1 mM sodium vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSF). Lysates were centrifuged to remove insoluble cellular matter and total protein concentration was measured with the Bio-Rad Protein Assay (Hercules, CA). Protein lysates were resolved on SDS-PAGE, transferred onto nitrocellulose

membranes and were subjected to western blotting using antibodies against total FAK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phosphorylated FAK (BioSourse International, Inc., Camarillo, CA), polyoma middle-T antigen (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and viculin (Sigma Chemical Company, St. Louis, MO).

Results

We have not encountered any difficulties during this phase of the investigation. Genotyping and immunoblotting results are reproducible.

We found that mammary carcinomas in the MMTV-PyVT female mice had increased FAK expression and constant expression of polyoma middle-T antigen expression. (Data not shown because it is comparable to data from positive control animals shown in Figure 4.)

b. Transgenic carcinogenesis – generating the target mice (MMTV-PyVT; FAK$^{Flox/Flox}$; MMTV-Cre).

a. Introduce the FAK$^{Flox/Flox}$; MMTV-Cre genotype into wild-type FVB mice.

b. Introduce the FAK$^{Flox/Flox}$ genotype into the MMTV-PyVT transgenic FVB mice.

c. Determine if back-crossing the MMTV-PyVT; FAK$^{Flox/Flox}$ animals to FVB background is needed for rapid (less than 3 months) mammary carcinogenesis and metastasis.

Rationale

Our MMTV-Cre FAK conditional knock-out animals are on a mixed background, whereas the MMTV-PyVT transgenic mice are on FVB background. First we started with two parallel mating routine (Steps 1A and 1B). In the 1A mating scheme a wild-type female FVB mouse was mated to a FAK$^{Flox/Flox}$; MMTV-Cre male mouse and female offspring with FAK$^{Flox/WT}$; MMTV-Cre genotype were used for subsequent mating. (We could not use FAK$^{Flox/Flox}$; MMTV-Cre female mice in this scheme, because the mammary gland in these females exhibit severe lobulo-alveolar hypoplasia which renders the dams unable to lactate). In the 1B mating scheme a female mouse with FAK$^{Flox/Flox}$ genotype was mated to a male MMTV-PyVT transgenic FVB mouse and male offspring with FAK$^{Flox/WT}$; MMTV-PyVT genotype was used in subsequent mating. In the next level of mating (Step 2) a FAK$^{Flox/WT}$; MMTV-Cre female was mated to a FAK$^{Flox/WT}$; MMTV-PyVT male. In this scheme target females (FAK$^{Flox/Flox}$; MMTV-Cre; MMTV-PyVT) were expected at 1/32 frequency, and positive control females (FAK$^{Flox/Flox}$; MMTV-PyVT) were expected at 1/32 frequency. To generate target and control mice in subsequent mating schemes we used the following mating pairs: FAK$^{Flox/Flox}$ female and FAK$^{Flox/Flox}$; MMTV-Cre; MMTV-PyVT male (3A) and FAK$^{Flox/WT}$ female and FAK$^{Flox/Flox}$; MMTV-Cre; MMTV-PyVT male (3B).

Since our target mice are of mixed background, we also started back-crossing schemes to generate animals with either FAK$^{Flox/Flox}$ or FAK$^{Flox/Flox}$; MMTV-Cre genotype on FVB background. These studies are on-going.

Materials and Methods

The PCR primers and conditions used in this step were described in section a. of Aim 1.

Results

We have not encountered any difficulties during this phase of the investigation. Genotyping results are reproducible.

So far we generated and partially analyzed the following animals: 3 positive control females (FAK$^{Flox/Flox}$; MMTV-PyVT) and 2 target females (FAK$^{Flox/Flox}$; MMTV-Cre; MMTV-PyVT).

c. Transgenic carcinogenesis – final study.

a. Compare tumor incidence and FAK expression and phosphorylation in both the test and control groups (necropsy, histology, immunohistochemistry, and Western blotting).

Rationale

We hypothesized that mammary-specific deletion of FAK would result in reduced tumor incidence and metastasis. Our hypothesis rests on two important *in vitro* observations concerning FAK signaling related to carcinogenesis. First, studies from our lab showed that FAK is indispensable for robust cell proliferation [12]. Moreover, FAK inhibits endocytosis of MT1-MMP (a membrane-bound matrix metalloproteinase) and thereby promotes degradation of the extracellular

matrix [13]. This latter observation is important for invasion of the surrounding tissue by the primary tumor and during the final phase of metastasis, when metastatic tumor cells colonize and invade distant organs.

It is possible, albeit unlikely, that target females will not develop mammary tumors. The more plausible possibility is, however, that these target females will develop mammary tumors, but these tumors will appear after longer latency, and/or will exhibit slower growth rate and/or metastatic spread. There are two possible explanations for the presence of mammary tumors in target animals (in which the mammary epithelium has very little functional FAK). The first possibility is that in these animals mammary tumors are initiated in mammary epithelial cells in which FAK was not deleted. The second possibility is that the tumors in the target mice start in mammary epithelial cells that have no functional FAK protein and the neoplastic transformation is achieved through alternative mechanisms, like activation of phosphatidylinositol 3-kinase/Akt pathway [19].

Materials and Methods

Female mice of desired phenotypes (positive control and target) were identified with PCR using previously described primers and conditions. Mice were observed and palpated weekly for the presence of mammary tumors starting at 8 weeks of age. Tumor size was measured using a caliper and size, as well as tumor number in each animal, was recorded. Tumor free interval, cumulative tumor burden, and tumor growth-rate were used to compare carcinogenesis in control and target group. Tumor-bearing female mice were euthanized 50 days after the appearance of the first tumor. Euthanasia was performed using CO_2. Sample collection for histology and protein extracts has been described previously in this report.

Histological examination of each animal included multiple sections of different mammary tumors, grossly normal areas of the mammary gland (if present), and major organs. To detect micrometastases in the lungs, lung lobes were sectioned at 3 mm intervals in cranio-caudal direction. Histologic confirmation of mammary carcinoma was performed by a veterinary pathologist (TN).

FAK, polyoma middle-T antigen expression and FAK phosphorylation were also assessed in mammary carcinomas from positive control and target females.

Results

To determine tumor-free interval, female mice were palpated weekly for the presence of subcutaneous mammary tumors. The number of animals without palpable subcutaneous mammary tumors was expressed as a ratio within their respective genotypic group. Animals in all genotypic categories (target and positive control) developed tumors. Positive control female mice ($FAK^{Flox/Flox}$; MMTV-PyVT, n=3) had grossly detectable mammary tumors at 71, 71, and 97 days of age, respectively (average tumor-free interval: 79.7 days). Target female mice ($FAK^{Flox/Flox}$; MMTV-Cre; MMTV-PyVT, n=2) had grossly detectable mammary tumors at 107 and 118 days of age, respectively (average tumor-free interval: 112.5 days). These data are summarized in Figure 1. So far 2 of the positive controls were euthanized 50 days after the appearance of mammary tumors. None of these animals had pulmonary metastasis. Two target mice were similarly euthanized 50 days after the appearance of mammary tumors. None of these animals had pulmonary metastasis either. Additionally, no mammary tumors were noted in negative control ($FAK^{Flox/Flox}$; MMTV-Cre or $FAK^{Flox/Flox}$) females within the observational period.

To determine cumulative tumor burden per animal in each genotypic group, female mice were palpated weekly for presence of subcutaneous mammary tumors and tumor diameter was measured with a caliper. The diameters of all tumors in each animal were added to arrive at cumulative tumor burden. The cumulative tumor burden is 2 to 3 times larger in positive control ($FAK^{Flox/Flox}$; MMTV-PyVT, n=3) animals than in target ($FAK^{Flox/Flox}$; MMTV-Cre; MMTV-PyVT, n=2) animals (Figure 2).

Daily growth rates of subcutaneous mammary tumors were calculated using cumulative tumor burden data within the distinct intervals of the observational period. The first interval is from age 71 days to age 96 days, the second one is from age 96 days to age 107 days, the third interval is from

age 107 days to age 118 days, and the fourth one is from age 118 days to age 132 days. Once animals in both groups developed tumors (second interval), both positive control and target females exhibited similar growth rates, 0.45 and 0.61, respectively. In the third interval, the daily tumor growth rate in the positive control females was twice than that in the target females (2.3 and 1.14, respectively). In the fourth interval tumor growth rates declined in both groups, most likely due to the systemic effects of the widespread tumor burden (manifested in severe emaciation) (Figure 3).

Mammary carcinoma lysates from positive control (n=2) and target (n=2) female mice were analyzed by immunoblotting for FAK and transgenic polyoma middle-T antigen expression and FAK phosphorylation (Figure 4). All of the mammary tumor lysates have abundant transgenic polyoma middle-T antigen expression. None of the mammary tumor lysates from target females have appreciable FAK expression or FAK phosphorylation, whereas there is abundant FAK expression in mammary tumor lysates from positive control females. One tumor sample from a positive control female shows minimal FAK phosphorylation.

b. Compare the rate of proliferation and apoptosis in the primary tumor and in the metastatic foci (if any) in both the control and test group.

Rationale

To account for clinical data (longer tumor free interval and slower tumor growth rate in target females) neoplastic cell turnover rate has to be determined. Cell turnover is ultimately determined by the balance of proliferation and apoptosis.

Materials and Methods

To assess cell proliferation rate, BrdU (5-Bromo-2′-deoxyuridine, Sigma-Aldrich, St. Louis, MO) was injected intraperitoneally into mice at 100 mg/kg body weight 3 hours prior to euthanasia. Euthanasia, necropsy, and tissue processing for histology have been described in detail in previous sections this report. Unstained tissue sections were deparaffinized in xylene, rehydrated in graded ethyl-alcohol solutions, and stained using a BrdU staining kit (Zymed® Laboratories, South San Francisco, CA) according to the manufacturer's instructions. Sections were observed under light microscope and BrdU-positive mammary carcinoma cells were identified by a veterinary pathologist (TN) and their number was expressed as a percentage of total tumor cells. Statistical analysis (two-tailed T-test) was performed using average percentages from three independent experiments and the difference between positive control and target proliferation rates was interpreted as significant when p value was below 0.05.

To assess apoptotic rate, unstained histologic sections were deparaffinized in xylene, rehydrated in graded ethyl-alcohol solutions, and stained using an ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Chemicon International Temecula, CA) according to the manufacturer's instructions.

Results

These studies are on-going; no preliminary data are available.

Bulleted List of Key Research Accomplishments

- **Mammary epithelial-specific deletion of Focal Adhesion Kinase (FAK) was introduced into MMTV-PyVT transgenic animals. We monitored mammary carcinogenesis in positive control ($FAK^{Flox/Flox}$; MMTV-PyVT) and target ($FAK^{Flox/Flox}$; MMTV-Cre; MMTV-PyVT) females.**

- **Average tumor free interval in the positive control animals is 79.3 days, whereas it is 112.5 days in the target animals, therefore tumor-free interval in target females was lengthened by 33.2 days.**

- **Cumulative tumor burden is always larger in positive control animals.**

- **Tumor growth rate is larger in the positive control animals.**

- **Transgenic polyoma middle-T antigen was abundant in mammary tumors from both positive control and target females, whereas FAK is not detected in mammary tumors from target animals.**

- **Based upon our data, we conclude that FAK, although not absolutely necessary, but it still plays a contributory role in polyoma middle-T antigen induced mammary carcinogenesis in mice.**

Reportable outcomes

Submitted manuscript

Authors: Tamas Nagy, Huijun Wei, Tang-Long Shen, Xu Peng , Chun-Chi Liang, Boyi Gan, and Jun-Lin Guan

Title: Mammary Epithelial-Specific Deletion of the Focal Adhesion Kinase Gene Leads to Severe Lobulo-Alveolar Hypoplasia and Secretory Immaturity of the Murine Mammary Gland

Target journal: *Molecular and Cellular Biology*

Conclusions

In this report we give a preliminary account on the consequences of mammary-specific Focal Adhesion Kinase (FAK) deletion in mammary-specific polyoma middle-T transgenic mice.

We monitored mammary carcinogenesis in positive control ($FAK^{Flox/Flox}$; MMTV-PyVT) and target ($FAK^{Flox/Flox}$; MMTV-Cre; MMTV-PyVT) females. We found that mammary-specific FAK deletion lengthens the tumor-free interval by 33.2 days. We also found that cumulative subcutaneous mammary tumor burden and tumor growth rate were larger in positive control females than in target females. Additionally we also found that FAK is virtually not expressed in mammary tumors from target animals, but FAK expression was abundant in positive control animals. Transgenic polyoma middle-T antigen expression was similar in mammary tumors from both positive control and target females.

Based upon the preliminary data, we conclude that FAK, although not absolutely required, but it still plays a contributory role in polyoma middle-T antigen induced mammary carcinogenesis in female mice. Studies are underway to confirm our preliminary data and identify the primary pathway that is ultimately responsible for mammary carcinogenesis in these genetically modified mice.

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Appendices

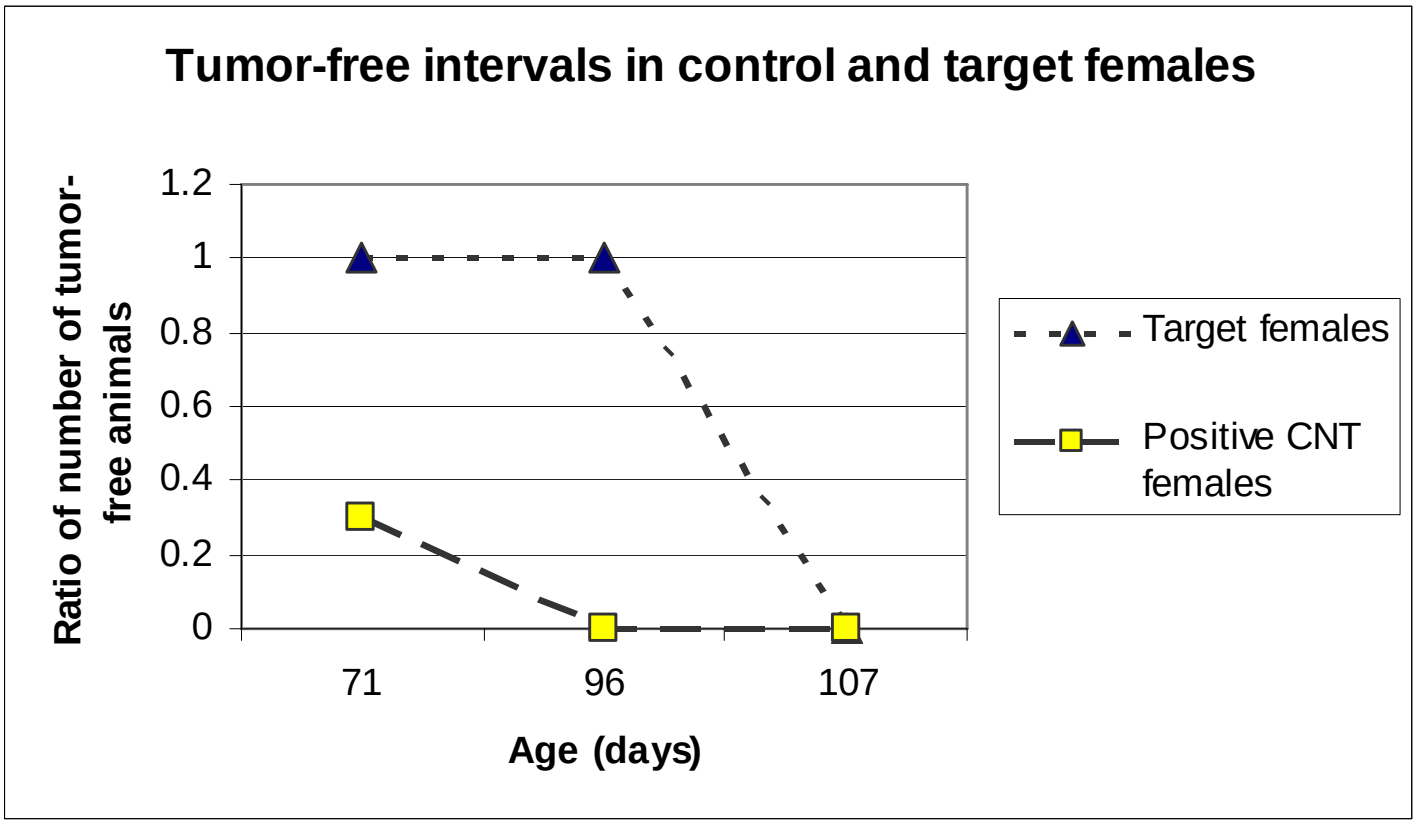

Figure 1. Tumor-free intervals in control and target female mice.
Female mice were palpated weekly for the presence of subcutaneous mammary tumors. The number of animals without palpable subcutaneous mammary tumors was expressed as a ratio within their respective genotypic group. Thirty-three per cent of positive control ($FAK^{Flox/Flox}$; MMTV-PyVT, n=3) females had no palpable subcutaneous mammary tumors by 71 days of age and all of them had such tumors by 96 days of age. On the other hand, 100 per cent of target ($FAK^{Flox/Flox}$; MMTV-Cre; MMTV-PyVT, n=2) females were tumor free at 96 days of age and only developed mammary tumors by 107 days of age. The difference between the earliest onset of tumor in positive control *versus* target females is 33.2 days. No mammary tumors were noted in negative control ($FAK^{Flox/Flox}$; MMTV-Cre or $FAK^{Flox/Flox}$) females within the observational period.

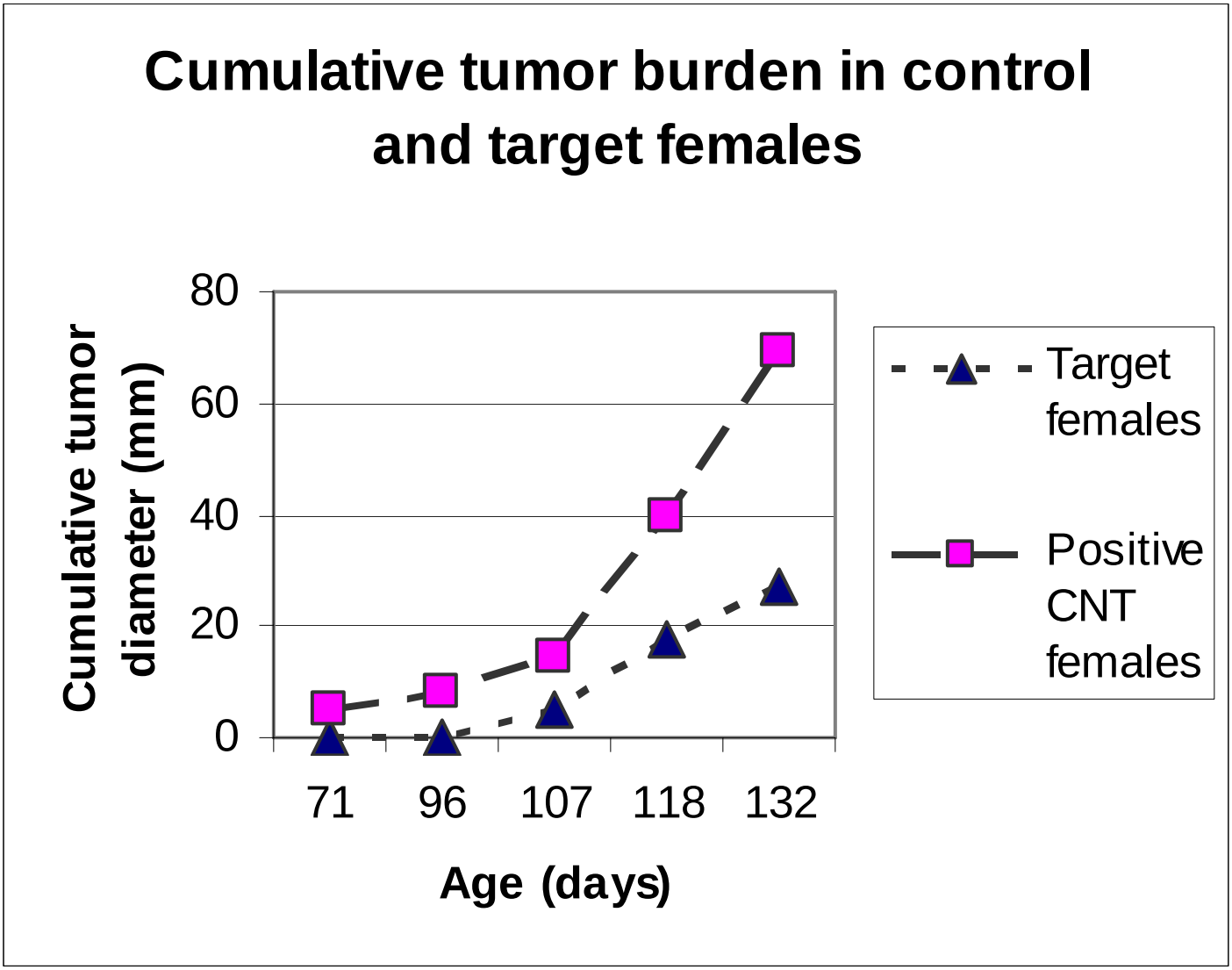

Figure 2. Cumulative tumor burden in control and target females.
Female mice were palpated weekly for presence of subcutaneous mammary tumors and tumor diameter was measured with a caliper. The diameters of all tumors in each animal were added to arrive at cumulative tumor burden (in millimeters). The cumulative tumor burden is 2 to 3 times larger in positive control ($FAK^{Flox/Flox}$; MMTV-PyVT, n=3) animals than in target ($FAK^{Flox/Flox}$; MMTV-Cre; MMTV-PyVT, n=2) animals.

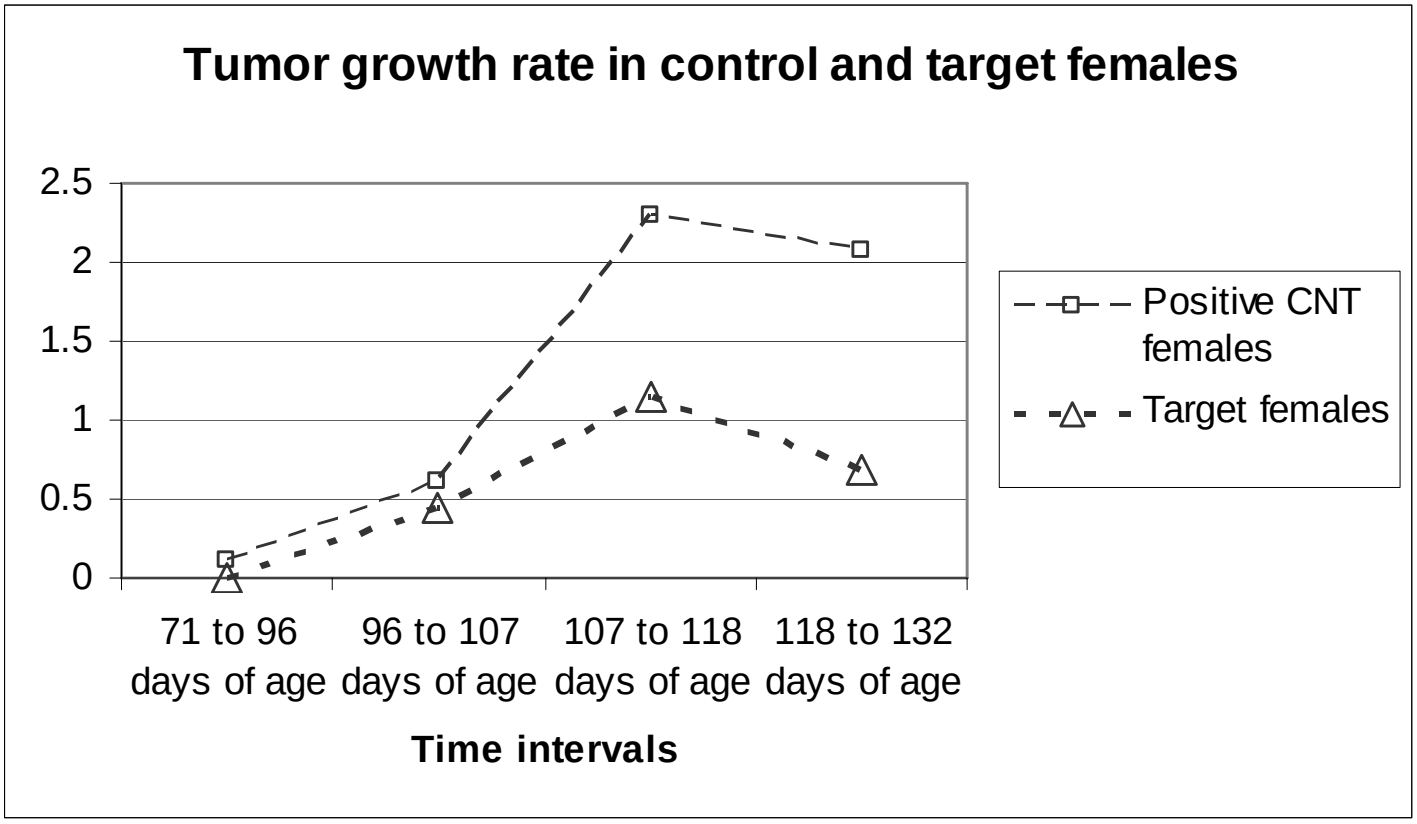

Figure 3. Growth rate of subcutaneous mammary tumors in control and target females.
Daily growth rates of subcutaneous mammary tumors were calculated using cumulative tumor burden data within the distinct intervals of the observational period. Once animals in both groups developed tumors (second interval), both positive control and target females exhibited similar growth rates, 0.45 and 0.61, respectively. In the third interval, the daily tumor growth rate in the positive control females was twice than that in the target females (2.3 and 1.14, respectively). In the forth interval tumor growth rates declined in both groups, most likely due to the systemic effects of the widespread tumor burden (manifested in severe emaciation).

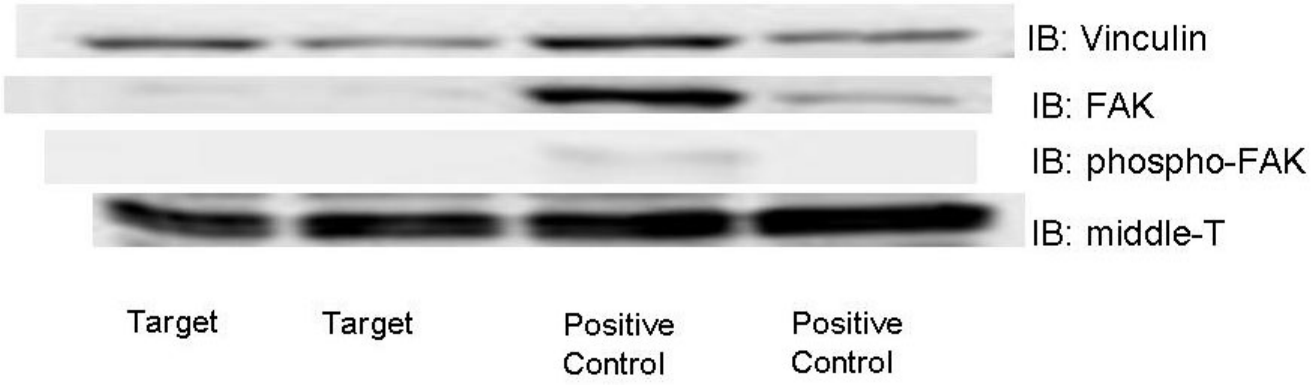

Figure 4. Immunoblotting for FAK, transgenic polyoma middle-T antigen expression and FAK phosphorylation.

Mammary carcinoma lysates from positive control (n=2) and target (n=2) female mice were analyzed by immunoblotting for FAK and transgenic polyoma middle-T antigen expression and FAK phosphorylation. All of the mammary tumor lysates have abundant transgenic polyoma middle-T antigen expression. None of the mammary tumor lysates from target females have appreciable FAK expression or FAK phosphorylation, whereas there is abundant FAK expression in mammary tumor lysates from positive control females. One tumor sample from a positive control female shows minimal FAK phosphorylation. Immunoblotting for vinculin serves as loading control.